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GENETIC TRANSFORMATION AND HYBRIDIZATION

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Expression of an *Arabidopsis CAX2* variant in potato tubers increases calcium levels with no accumulation of manganese

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Abstract Previously, we made a chimeric *Arabidopsis thaliana* vacuolar transporter CAX2B [a variant of N-terminus truncated form of CAX2 (sCAX2) containing the "B" domain from CAX1] that has enhanced calcium (Ca^{2+}) substrate specificity and lost the manganese (Mn^{2+}) transport capability of sCAX2. Here, we demonstrate that potato (*Solanum tuberosum* L.) tubers expressing the CAX2B contain 50–65% more calcium (Ca^{2+}) than wild-type tubers. Moreover, expression of *CAX2B* in potatoes did not show any significant increase of the four metals tested, particularly manganese (Mn^{2+}). The *CAX2B*-expressing potatoes have normally undergone the tuber/plant/tuber cycle for three generations; the trait appeared stable through the successive generations and showed no deleterious alternations on plant growth and development. These results demonstrate the enhanced substrate specificity of CAX2B in potato. Therefore, CAX2B can be a valuable tool for Ca^{2+} nutrient enrichment of potatoes with reduced accumulation of undesirable metals.

Keywords *Arabidopsis* · Calcium · H^+/Ca^{2+} antiporter · Manganese · Potato

Abbreviation *CAX*: Cation exchanger

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Introduction

Potatoes are an important agricultural commodity and are widely consumed worldwide; however, most potatoes contain little calcium (Ca^{2+}). The increase in the bioavailability of Ca^{2+} in vegetables could help enhance its nutritional value and also improve yield (Marschner 1995). One molecular-genetic approach to alter Ca^{2+} levels in plants is to engineer a high expression of Ca^{2+} transporters and Ca^{2+}-binding proteins (Hirschi 1999; Jang et al. 2003; Wyatt et al. 2002). The ectopic expression of deregulated *CAX1* (termed *sCAX1*, missing the N-terminal autoinhibitor) in various plants increases Ca^{2+} levels but it is known to induce Ca^{2+} deficiency-like symptoms due in part to a significant decrease in cytosolic Ca^{2+} level as the result of the sequestration of the ion into the vacuole (Hirschi 1999; Kim et al. 2005; Park et al. 2004, 2005a,b). Biochemical and genetic analyses have confirmed that CAX1 is a high capacity, low affinity Ca^{2+}/H^+ antiporter and an important component of Ca^{2+} homeostasis in the plant (Cheng et al. 2003). CAX2, however, has a lower capacity and affinity for Ca^{2+} transport than CAX1 and it also can transport other metals such as Mn^{2+} (Hirschi et al. 2000). In tobacco, even though *CAX2*-expressing tobacco plants accumulated Ca^{2+} comparable to those seen in *sCAX1*, these plants were, for the most part, as vigorous as the controls (Hirschi et al. 2000).

Recently, Shigaki et al. (2003) identified specific domains in the N-terminus truncated (active) CAX2 (sCAX2) that mediate Mn^{2+} substrate specificity and altered these domains to abolish the Mn^{2+} transport capability of CAX2 in yeast. Among the six sCAX2 mutants made by site-directed mutagenesis, three mutants, namely, CAX2A, CAX2B, and CAX2C showed complete loss of Mn^{2+} transport. In addition, CAX2B, which contains amino acids 150–160 of CAX1, showed a significantly reduced activity in Ca^{2+} transport as compared to sCAX1 or sCAX2. Considering the past report on Ca^{2+} deficiency symptoms caused by ectopic expression of *sCAX1* in some plants, this mutant could be a valuable tool for crop improvement with specific Ca^{2+} enrichment with reduced accumulation of untargeted metals.

Here, we report the generation and analysis of transgenic potatoes that express the sCAX2 mutant CAX2B and its potential impact on plant growth. We also investigated the effect of *CAX2B* expression in potatoes as a method to determine if utilizing the s*CAX2* mutant expression may be a means in reducing any potential deleterious phenotypes due to excessive increase in vacuolar Ca^{2+} level and transport Mn^{2+}. This study suggests that the modulation of Ca^{2+} transporters could make an important contribution toward increasing the value of various important agriculturally crops.

Materials and methods

Plant materials, transformation and growth conditions

The transformation of potatoes (*Solanum tuberosum* L. var Daejiree) was performed via the *Agrobacterium*-mediated transformation by using internode explants. A 5 mm internodes from potatoes, after 6 weeks growth in vitro, were excised and cultured on a MS medium (Murashige and Skoog 1962) with 100 mg/l inositol, 3% (w/v) sucrose, 2 mg/l zeatin (Sigma-Aldrich, http://www.sigmaaldrich.com), 0.3 mg/l indole acetic acid (IAA) (Sigma-Aldrich, http://www.sigmaaldrich.com) and 0.8% (w/v) Plant Agar™ (Duchefa, http://www.duchefa.com). After preculturing for 1 day, the internode explants were dipped in an *Agrobacterium* culture, blotted and re-cultured on the same media for 72 h. Internode sections were then cultured on a MS selection medium containing 3% (w/v) sucrose, 100 mg/l inositol, 2 mg/l zeatin, 0.3 mg/l IAA, 100 mg/l kanamycin monosulfate (Sigma-Aldrich, http://www.sigmaaldrich.com), 250 mg/l Clavamox®, and 0.8% (w/v) Plant Agar™. The shoots that developed on this medium were excised when they were at a length of 1–2 cm and transferred to a MS medium with 3% (w/v) sucrose, 100 mg/l kanamycin monosulfate, 250 mg/l Clavamox®, and 0.8% (w/v) Plant Agar™ for rooting. Cultures were maintained at 25°C under cool-white fluorescent (50–60 $\mu E\ m^{-2}\ s^{-1}$) light with a 16 h photoperiod. After 6–8 weeks, the plant regenerated roots were subcultured on MS medium with 9% sucrose for microtuberization, and the other plants transferred to a greenhouse and grown in 3-l pots filled with commercial compost. Plants were regularly watered, and once a month they were fertilized with a liquid half-strength MS medium. The temperature of the greenhouse was maintained between 20°C and 23°C. The tubers were harvested when the plants started to senescence. The first-generation tubers (primary *CAX2B*-expressing lines) from the potato derived tissue culture explants were rinsed free of soil, and then stored at 4°C until shoots started to develop. Tubers of similar size were then planted and grown in 3-l pots filled with commercial compost. Their successive-cloned generations were produced in a greenhouse under the same growth conditions as described above.

Bacterial strain and plasmid

The coding region of *CAX2B* was cloned into pBIN19 (CLONTECH Laboratories, http://www.clontech.com), which contained the 35S promoter and *nos* terminator (Hull et al. 2000). The plasmids pCaMV35S::*CAX2B* was introduced into *Agrobacterium tumefaciens* strain LBA4404 (octopine, Hoekema et al. 1983) using the freeze-thaw method (Holsters et al. 1978).

DNA isolation, PCR and Southern blot analysis

Potato genomic DNA was isolated from the newly developing young leaves of acclimated plants in a greenhouse using the DNeasy Plant kit™ (Qiagen, http://www.qiagen.com). For PCR amplification of *nptII* gene, one set of primers were used in this study. These were 5′-GAGGCTATTCGGCTATGACTG-3′ for primer 1; and 5′-ATCGGGAGCGGCGATACCGTA-3′ for primer 2. The amplification of *nptII* involved 35 cycles at 95°C for 30 s, at 70.5°C for 45 s, and at 72°C for 1 min. For a Southern blot analysis, DNA (10 μg) was digested with *Xba* I (which made only one cut in the T-DNA region and another cut elsewhere in the plant DNA) and electrophoresed in a 0.8% agarose gel. The DNA was then transferred to a nylon membrane (Zeta-Probe GT membrane, BIORAD Laboratories, http://www.bio-rad.com) using capillary blotting. A 1.2 kb fragment of the *CAX2B* gene was extracted from the gel, labeled with ^{32}P, and used as a probe for Southern blot analysis. Blotting, labeling, hybridization, and washing were carried out according to the manufacturer's instructions.

RNA isolation and Northern blot analysis

Total RNA was extracted from fresh potato tuber tissue using RNeasy Plant Kits (Qiagen, http://www.qiagen.com) according to the manufacturer's instructions. Northern analysis was carried out as reported by (Hirschi 1999). Total RNA (10 μg) was separated on a 1.2% agarose gel containing 1.5% formaldehyde and blotted onto a Zeta-Probe GT membrane (BIORAD Laboratories, http://www.bio-rad.com) according to the manufacturer's instructions. Hybridization and washing were

conducted as previously described in Southern blot analysis.

Calcium and mineral analysis

Harvested potato tubers and leaves were dried at 70°C for 4 days and a total of 0.25 g (dry weight) from the potato tubers and leaves were digested as reported by (Feagley et al. 1994). Total Ca^{2+} and mineral content per gram of dry weight was determined by inductively coupled plasma emission spectrophotometry (Spectro, http://www.spectro.com).

Results

CAX2B-expression in potato

In previous work, CAX2B was partially characterized for its ability to suppress defects in vacuolar Ca^{2+} transport in yeast. CAX2, however, appears to have biochemical properties in yeast that are inconsistent with its involvement in transport of Ca^{2+} into the vacuole (Shigaki et al. 2003). In order to examine the role of CAX2 in Ca^{2+} homeostasis, we expressed *CAX2B*, which was driven by the cauliflower mosaic virus 35S promoter (35S) in potatoes (Fig. 1a). We have generated 31 *CAX2B*-expressing independent lines. First, we examined the presence of the transgenes by PCR analysis. It demonstrated that all 31 independently generated primary transgenic plants possessed a stable intergrated gene for kanamycin resistance (*nptII*) (Fig. 1b). We randomly selected and confirmed five transgenic potato lines by Southern blot analysis. As demonstrated in Fig. 1c, the transgenic potato lines contain various copy numbers of the *CAX2B* expression vector. The lines we have termed T3 and T5 appear to contain single insertions. RNA gel blot documents that the *CAX2B* transcripts accumulated in all of the transgenic lines (Fig. 1d, parts of the data are shown). The inability to detect *CAX2B* homology in the control lines by Southern or Northern analysis

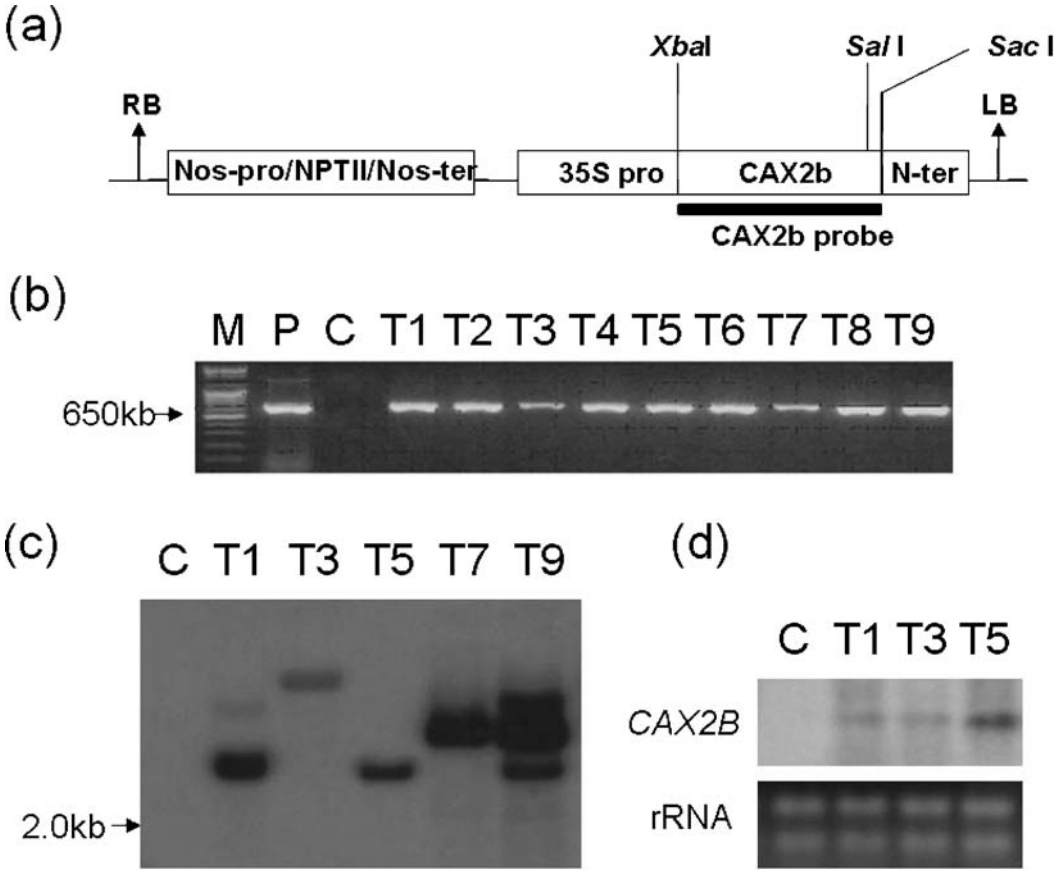

Fig. 1 Molecular analyses of primary transgenic potato plants. (**a**) T-DNA regions of pCaMV35S::CAX2b. Abbreviations: RB, right border; LB, left border; Nos-pro, nopaline synthase promoter; NPTII, neomycin phosphotransferase; Nos-ter, nopaline synthase terminator; CAX2b, cation exchanger 2 mutant; N-ter, nopaline synthase terminator; 35S-pro, cauliflower mosaic virus (CaMV) 35S promoter. (**b**) PCR detection of *nptII* genes in the putative transgenic potato plants. Expected 650 bp fragments of *nptII* were amplified in all tested plantlets after acclimatization. *Lanes M*, molecular size marker; *P*, positive control (plasmid DNA); *C*, negative control (wild-type potato); *T1* through *T9*, transgenic potatoes with pCaMV35S::*CAX2b*. (**c**) Southern blot analysis of the transgenic potatoes. Ten micrograms of potato genomic DNA were digested with *Xba* I and hybridized with the *CAX2b* cDNA probe. *Lanes C*, wild-type potato; *T1*, *T3*, *T5*, *T7*, and *T9*, transgenic potatoes with pCaMV35S::*CAX2b*. (**d**) Northern blot analysis of the transgenic potatoes. Ten micrograms of total RNA from potato tubers were hybridized with the *CAX2b* cDNA probe. Ethidium bromide stained rRNA (*bottom*) is shown as a loading control

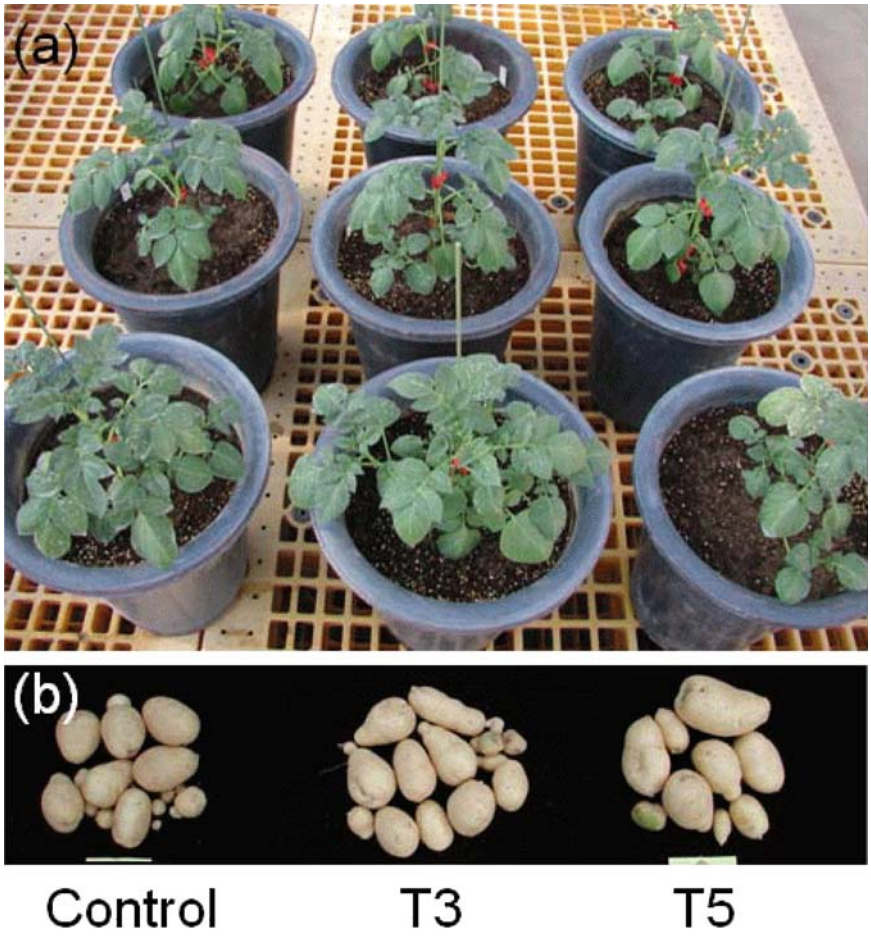

Fig. 2 The phenotype of transgenic *CAX2B*-expressing primary potatoes. (**a**) The morphology and growth characteristics of the *CAX2B*-expressing potatoes (*middle* and *right*) are indistinguishable from that of the wild-type controls (*left*). (**b**) The yield and size distribution of the *CAX2B*-expressing potatoes (T3 and T5) are indistinguishable from that of the wild-type controls (control)

may be due to the stringency of hybridization used in this study. Three primary transgenic lines showing single-copy (T3 and T5) and low-copy (T1) from the Southern analysis were selected and subjected to further analysis of Ca^{2+} accumulation in the *CAX2B*-expressing second-generation tubers.

Phenotypes of *CAX2B*-expressing potatoes

While the deregulated *sCAX1*-expressing plants showed Ca^{2+} deficiency-like symptoms (i.e. necrotic lesions) that were suppressed by the addition of Ca^{2+} (Hirschi 1999; Park et al. 2004, 2005a), the *CAX2B*-expressing potatoes did not alter their morphology or growth characteristics (Fig. 2a). In addition, the *CAX2B*-expressing potatoes did not require any additional Ca^{2+} supplementation for normal growth. The total yield (as measured by the fresh weight of the potato tubers) of the primary *CAX2B*-expressing lines was indistinguishable from that of the controls (Figs. 2b and 3). The variation in yield within the *CAX2B*-expressing lines was not significantly different from that within the control lines. Furthermore, the yields of the second- and third-generation *CAX2B* lines also did not differ from those of the controls (Fig. 3).

The accumulation of calcium and manganese in *CAX2B*-expressing potato plants

The total accumulation of Ca^{2+} and Mn^{2+} was measured in the edible (tubers) and aerial (leaves) portions of the

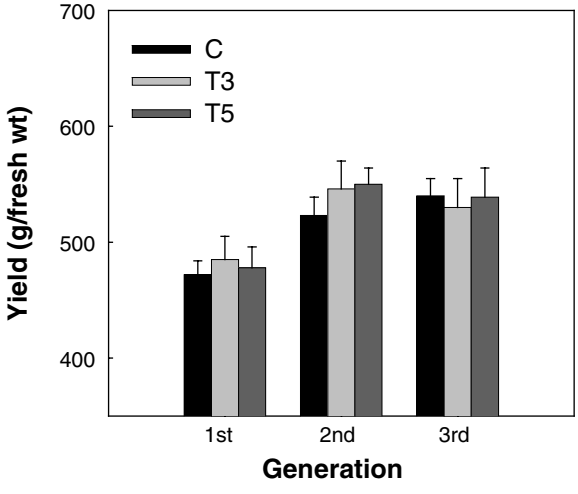

Fig. 3 The yield of the primary, second- and third-generation *CAX2B*-expressing potatoes (T3 and T5) and wild-type controls (control). Data are presented as means ± SD of measurements for the fresh weight of total potato tubers from 8 T3, 8 T5 and 6 control lines

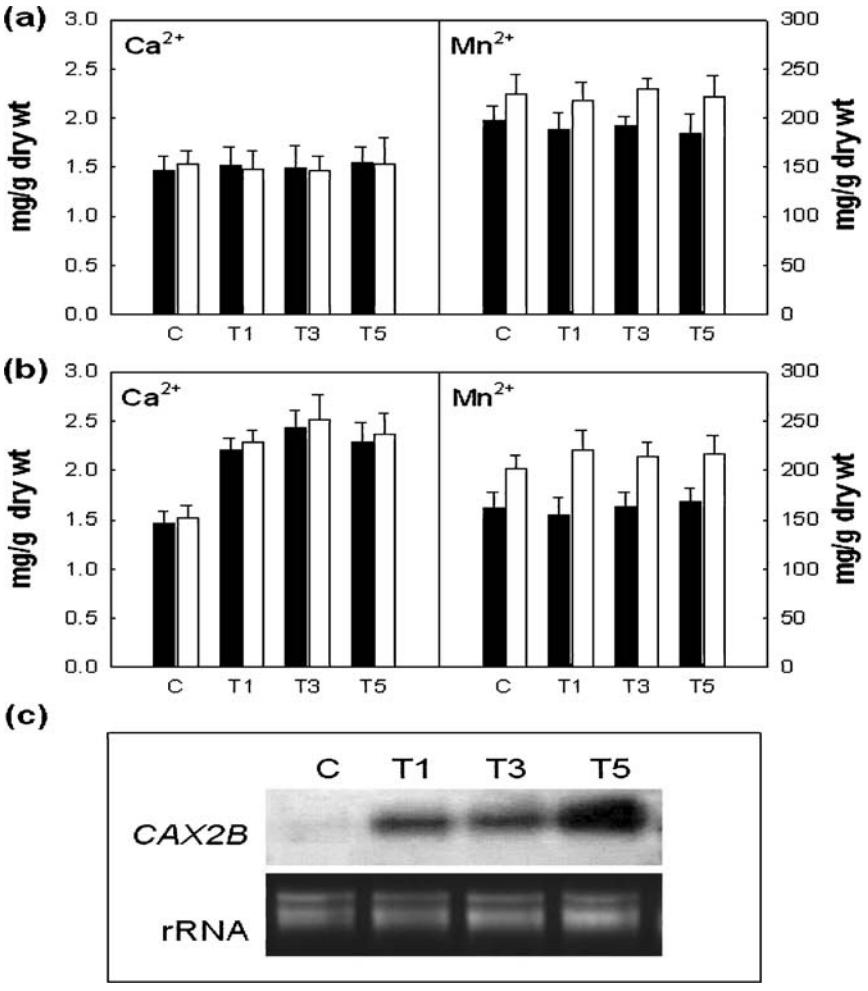

Fig. 4 The Ca^{2+} and Mn^{2+} accumulation levels in the primary and second-generation *CAX2B*-expressing potato (T1, T3 and T5) and wild-type controls (control). The total Ca^{2+} and Mn^{2+} content of potato leaves (**a**) and tubers (**b**) were determined by an inductively coupled plasma emission spectrophotometer. *Black bars* represented the primary transgenic *CAX2B*-expressing potato and *whiter bars* represented the second-generation transgenic *CAX2B*-expressing potato. (**c**) Northern blot analysis of the second-generation *CAX2B*-expressing potatoes. *Lanes C*, wild-type potato; *T1*, *T3*, and *T5*, transgenic potatoes with pCaMV35S::*CAX2b*. Ten micrograms of total RNA from potato tubers were hybridized with the *CAX2b* cDNA probe. Ethidium bromide stained rRNA (*bottom*) is shown as a loading control

potatoes. As shown in Fig. 4, the *CAX2B*-expressing potato tubers contained significantly more Ca^{2+} (50–65% increase) than the controls, whereas no significant increase of Mn^{2+} was observed in any of the lines analyzed (Fig. 4b). In the potato leaves, there was no significant increase observed of Ca^{2+} or Mn^{2+} in any of the lines analyzed (Fig. 4a). We were also interested in determining if the *CAX2B*-expressing potatoes also showed increases in other minerals. There was no significant increase observed of any other minerals (Cu^{2+}, Fe^{2+}, and Zn^{2+}) in the tubers of the *CAX2B*-expressing lines, as compared to the control lines (Fig. 5).

In order to confirm whether the increase Ca^{2+} accumulation was consistent in the next-generation tubers, second-generation tubers derived from the first-generation potato tubers were tested. Initially, RNA gel blot analysis confirmed that the *CXA2B* transcripts accumulated in all of the second-generation tubers (Fig. 4c). As shown in the primary *CAX2B*-expressing potatoes, all of the *CAX2B*-expressing second-generation potatoes contained significantly more Ca^{2+} (50–65% increase) in the tubers than in the controls, whereas there was no significant increase of Mn^{2+} in any of the lines analyzed (Fig. 4b). In the potato leaves, there was no significant increase of Ca^{2+} and Mn^{2+} in any of the second-generation lines analyzed (Fig. 4a). In addition, there was no significant increase in any other minerals (Cu^{2+}, Fe^{2+}, and Zn^{2+}) in the *CAX2B*-expressing second-generation lines as compared to the control lines (data not shown).

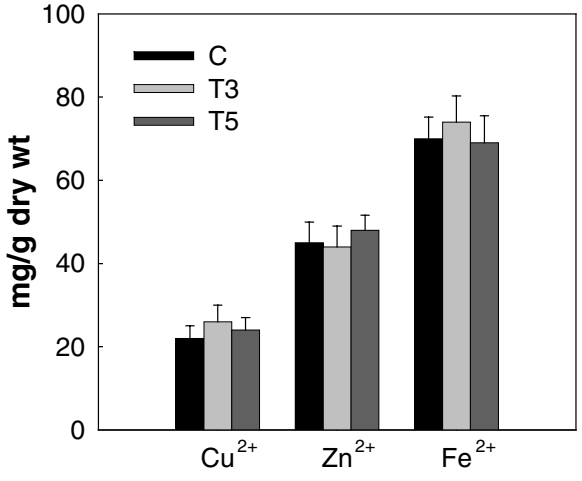

Fig. 5 The total mineral (Cu^{2+}, Fe^{2+}, and Zn^{2+}) content of the primary transgenic *CAX2B*-expressing potato tubers. Data are presented as means ± SD of the 8 T3, 7 T5, and 4 control lines (five individual replications per line)

Discussion

The manipulation of CAX transporters, through alteration of the expression and substrate specificity, is an essential component in developing plants with proper increases of Ca^{2+} levels or in removing some Ca^{2+} deficiency-like symptoms due to excessive sequestration of Ca^{2+} into the vacuole. In previous studies, the expression of *sCAX1* has been shown to dramatically increase the total Ca^{2+} accumulation in the plants, but this also causes severe changes in growth and altered morphology (Hirschi 1999; Park et al. 2005b). In carrots and potatoes, the expression of *sCAX1* has been shown to increase the total Ca^{2+} accumulation in aerial portion of the plants, but this causes modest changes in altered morphology as well (Park et al. 2004, 2005a). The expression of *sCAX2* in tobacco and expression of full-length *CAX4* in tomato, however, did not appear to alter plant growth. This may be in part to low Ca^{2+} sequestration into the vacuole (Hirschi et al. 2000; Park et al. 2005b). However, potentially deleterious effects of sCAX2 expression have not been investigated in most plants. In this study, we selected a sCAX2 variant CAX2B for the Ca^{2+} enrichment of potato because it is less likely to cause growth defects than sCAX2, and yet it is likely to accumulate Ca^{2+} significantly. We have developed multiple *CAX2B*-expressing potato lines with various copies of the transgene (Fig. 1). The vast majority of these plants are healthy and indistinguishable from wild-type controls with regard to growth characteristics or tuberization (Fig. 2a, b). These results indicated that the expression of *CAX2B* in potato plants have no adverse effect on shoot growth and tuberization. Similar results have been observed in the transgenic potato plants expressing human β-amyloid protein (Andersson et al. 2003; Kim et al. 2003). In addition, the *CAX2B*-expressing potato lines displayed up to 65% more Ca^{2+} in tubers, and most importantly, this Ca^{2+} increase appears to be inherited in successive generations (Fig. 4b). Our findings suggest that the use of *CAX2B* eliminates some of the most severe or any modest symptoms of Ca^{2+} deficiencies that are associated with the expression of the high capacity Ca^{2+} transporter, *sCAX1* (Hirschi 1999; Park et al. 2004, 2005a,b).

CAX2 is 43% identical to CAX1 at the amino acid level and previous research suggests that the mutation of a discrete amino acid domain in this transporter confers Mn^{2+} transport in yeast assays (Shigaki et al. 2003). When *CAX2B* was expressed in yeast, it was a more specific Ca^{2+} transporter than other CAX2 mutants. The Ca^{2+} antiporter activity of CAX2B was 33.4% of that of CAX2, and the Mn^{2+} transport could not be detected (Shigaki et al. 2003). In this study, we have observed that the *CAX2B*-expressing potatoes only showed increase in the Ca^{2+} levels without an accumulation of Mn^{2+} (Fig. 4a, b), suggesting that the s*CAX2* mutant, *CAX2B*, could be used to accumulate higher levels of Ca^{2+}, without accumulating untargeted metals, especially Mn^{2+}.

There are well known relationships between divalent cation concentrations in plants such that increasing the levels of one can compete for divalent cation-binding sites, thus reducing the uptake of the amount of other minerals (Marschner 1995). We have also shown in previous work that the CAX transporters can translocate multiple cations. This study suggests there was no significant change in any other minerals with the *CAX2B*-expressing potato lines that were analyzed (Fig. 5). Given the emerging data regarding the various biochemical properties of the CAX transporter (Pittman and Hirschi 2003), however, the *CAX2B*-expressing plants will need to be grown and analyzed in a variety of growth conditions.

As outlined in the introduction, in terms of human nutrition, no single food source will rectify deficiency of Ca^{2+} intake. We have analyzed how ectopic expression of an *Arabidopsis* Ca^{2+} transporter *CAX2B*-expressing in potato plants driven by the CaMV 35S promoter consistently increases Ca^{2+} content without accumulation of unwanted metal, Mn^{2+}. Plants vary greatly in the amount of bioavailable Ca^{2+}, which represents the amount that can be digested, absorbed and metabolized (Marschner 1995). We will need to conduct feeding studies, however, to assess the bioavailability of potato tubers. Future studies will also need to demonstrate if the increases in Ca^{2+} in the *CAX2B*-expressing potatoes might alter other characteristics as a source of food such as the texture of potatoes. Our goal here is not to make potatoes a superior source of Ca^{2+}, but rather to improve an important staple food that is popular worldwide.

In conclusion, we have demonstrated the ability to modestly increase Ca^{2+} levels in potato tubers but not to accumulate unwanted metals through the heightened activity of a vacuolar transporter CAX2B, thereby boosting the bioavailability of Ca^{2+} in potatoes.

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